Localization and Stability of Technetium-99m-Sn-Pyrophosphate in Rat Neutrophils

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Technetium-99m has been suggested as an alternative radiolabel for white cells, and while its physical characteristics are nearly ideal, its stability and site of localization in this procedure are unclear. We examined these parameters by radiolabeling 10^8 neutrophils from rat peritoneum with 74 to 370 MBq technetium-99m-Sn-pyrophosphate. We found that the percentage of initial activity bound to neutrophils was quite variable, possibly because the radiolabel associated with several subfractions: 19.8 ± 11.5% (mean ± s.d.) with nuclei and plasma membranes, 25.6 ± 3.9% with mitochondria, 26.6 ± 9.8% with microsomes, and 29.2 ± 6.9% with cytosol. Approximately 80–90% of the radioactivity associated with neutrophils was not bound to protein and only about one-half of the activity localized to cell membranes was removable over 4 hr by pepsin digestion. We concluded that the variable labeling efficiency was due to the radiolabel's rather loose association with several cellular subfractions rather than specific binding to a unique substrate.

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Over the past several years the technique of radiolabeling white blood cells has become increasingly useful for investigating abscess detection (*1,2*) and cell trafficking (*3*). A number of workers have reported the use of technetium-99m (^{99m}Tc) as a radiolabel for neutrophils (*1,2–6*). A major advantage to using this radiolabel is that the metal chelators oxine and tropolone, which are necessary for labeling with indium-111 (^{111}In) and which have both been implicated in cell toxicity, can be avoided. In addition, use of ^{99m}Tc permits radiolabeling with higher levels of radioactivity and consequently greater photon fluence which allows shorter imaging times and lower radiation doses per unit of radioactivity.

One major problem hampering the progress of this procedure has been the inconsistent labeling yields achieved with many of the methods. One procedure frequently reported in the literature incorporates stannous pyrophosphate, but while relatively easy to perform, it results in wide variation in reported labeling efficiency—ranging from 20.5% to 81%.

A second significant problem has been the lack of information concerning such basic parameters as the site of localization of the radionuclide and the rate of loss of the radiolabel. We undertook the present study in an effort to understand the reasons for the inconsistent yields when neutrophils are radiolabeled with ^{99m}Tc.

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MATERIALS AND METHODS

Isolation of Neutrophils

Neutrophils obtained from the peritoneum of Sprague-Dawley rats were selected as the experimental model because of the ready availability of cells from this source. We tested several of the methods reported in the literature (*1,7*) for isolating rat neutrophils and finally selected the method of Hodinka and Modrzakowski with modification (*8*). For a given isolation, a male rat weighing between 200–400 g was injected intraperitoneally with 30 ml of 0.01% oyster glycogen (Sigma Chemical Company, St. Louis, MO 63178) dissolved in sterile normal saline. Eighteen hours later the animal was sacrificed with ether, after which 30 ml of normal saline containing 5 μl/ml heparin (1,000 U/ml) was injected into the peritoneal cavity. The rat's abdomen was then shaved and massaged and the peritoneal cavity was opened. Peritoneal exudate was collected into a 50-ml culture tube and sieved through a 4 in. × 4 in. sterile gauze sponge to remove large aggregates. The exudate was then centrifuged at 400 *g* for 10 min at 4°C, and pelleted cells were washed once with 10 ml of normal saline. Typically, a differential Wright Stain (Sigma Chemical) smear demonstrated greater than 85% polymorphonuclear leukocytes (PMN's) in the sample. If the sample was contaminated with erythrocytes, they were removed by

hypotonic lysis in 9 ml of sterile water accompanied by gentle mixing for 20 s. Neutrophils were recovered with one ml of 10x concentrated Hank's balanced salt solution (HBSS).

Radiolabeling Technique

Isolated rat neutrophils were radiolabeled with ^{99m}Tc using the method of Farid et al. (*1*). Approximately 1×10^8 neutrophils were incubated with mild agitation in 3 ml of saline containing 6 mg sodium pyrophosphate and 1.7 mg stannous chloride (TechneScan PYP, Mallinckrodt, Inc) for 15 min in a water bath at 37°C, after which they were washed once by centrifugation at 400 *g* for 10 min. The pellet was resuspended in one ml of saline containing 2–10 mCi (74–370 MBq) of [^{99m}Tc]pertechnetate and incubated for 10 min at room temperature. The cells were again centrifuged and washed once with 3 ml saline, and the labeling efficiency was determined as the fraction of total activity retained by cells.

Viability and Functional Ability of Radiolabeled Neutrophils

The viability of rat neutrophils was determined by using the trypan blue exclusion test (*9*) on an aliquot of cells hourly from 1 to 4 hr following incubation.

The retention of functional activity of the neutrophils was assessed by determining their ability to phagocytyze *Staphylococcus aureus* cells (*10,11*). Lag-phase *S. aureus* cells were added to 4×10^6 neutrophils (either radiolabeled or unlabeled control) and the mixtures were diluted with sufficient normal saline so that ~300 bacteria and 200 neutrophils per 100 μl were plated on agar and incubated at 37°C. The difference between the average number of surviving colonies at time zero and the average number in the 60-min plates yielded the number of colonies phagocytized by functional neutrophils.

Loss of radiolabel

The rate of loss of radioactivity from neutrophils was determined by dialyzing the labeled cells against variable concentrations of EDTA in 0.01*M* phosphate buffered saline, pH 7.4 (PBS). One milliliter of ^{99m}Tc-labeled neutrophils (~ 2×10^5 cells) was dialyzed against one liter of a bath consisting of either PBS alone or this buffer containing 0.005*M*, 0.01*M*, or 0.025*M* EDTA. In preliminary dialysis studies, solutions of the higher concentrations of EDTA in PBS used in this study were found to be significantly more effective at extracting ^{99m}Tc-Sn-pyrophosphate than was PBS alone. At times 0, 30 min, and 1, 2, 3, and 4 hr following the beginning of dialysis, a 1-ml aliquot of each dialysate was removed and counted for ^{99m}Tc activity. Counts were corrected for decay, adjusted for total dialysate volume, and reported as percentage of total activity in the dialysis bag.

Determination of Site of Technectium-99m Localization

The method of Ando et al. (*12*) was modified to determine the site of localization of ^{99m}Tc-Sn-pyrophosphate in neutrophils. The cells were suspended in physiological saline and disrupted with a Bronson ultrasonic probe for 60 sec, after which subcellular components were isolated by centrifugation. Specifically, nuclei and plasma membranes were separated at 3,000 *g* for 15 min; mitochondria at 5,000 *g* for 15 min; and microsomes at 105,000 *g* for 60 min. Radioactivity of both pellets and supernatants was determined, and the percentage of ^{99m}Tc in each component was calculated.

To determine if ^{99m}Tc was bound to protein, the pellets were resuspended in one ml of 0.1% Triton X-100 to solubilize membranes. Protein in each fraction was precipitated by adding 1.5 ml of cold 10% trichloroacetic acid (TCA), and the ^{99m}Tc content of both the supernatant and pellets was determined.

RESULTS

Isolation of Neutrophils

The modified method of Hodinka and Modrzakowski (*8*) for isolating neutrophils provided the highest number of cells per rat of any of the methods tested. Specifically, in over 25 isolations we normally obtained ~10^8 cells per animal, while other methods we tested yielded only 6×10^6 to 1×10^7 cells. This method also allowed us to eliminate the lysis step unless the sample was contaminated with erythrocytes from blood vessels in the peritoneal wall.

Radiolabeling Technique

Radiolabeling neutrophils with the ^{99m}Tc-Sn-pyrophosphate method produced inconsistent results. For example, the percentage of ^{99m}Tc bound to neutrophils was quite variable and ranged from 20%–70% (45.05 ± 25.88% mean ± s.d.).

Viability and Functional Ability

In six separate studies the mean viability for radiolabeled cells was found to be 92.8 ± 4.1% following isolation, and decreased to 82.6 ± 4.3% over 4 hr. The viability for unlabeled cells was determined in one study and, over the same time period, ranged from 91.3% to 87.3%.

Four separate tests of bactericidal ability showed that unlabeled control neutrophils killed 42.7% of about 80 initial colonies, while an identical number of neutrophils radiolabeled with ~2–10 mCi (74-370 MBq) of ^{99m}Tc killed 41.3% of 77 initial colonies. Thus, radiolabeling at this level of activity did not adversely affect either viability or functioning of neutrophils.

Loss of Radiolabel

We found that a small fraction of the ^{99m}Tc associated with neutrophils following radiolabeling by the Sn-pyrophosphate method seems to be unbound or only loosely bound to the cells. As can be seen in Figure 1, ~10% of the initial activity could be removed over 4 hr by simply dialyzing against physiological buffer. This is similar to the results of Uchida and Vincent (*4*), who suspended leukocytes in HBSS at pH 7.4, and found that 92.2 ± 2.1% (mean ± s.e.m.) of the ^{99m}Tc remained with the cells at 5 hr. In our study EDTA, at concentrations up to 0.025*M*, was not effective in removing any additional amounts of ^{99m}Tc from the cells, presumably indicating the ^{99m}Tc is affixed to some cell component from which it slowly elutes. Over a 4-hr period, the net loss (4-hr value minus 0-hr value) for the control preparation (no EDTA) was 10.6 ± 6.4% (mean ± s.d.),

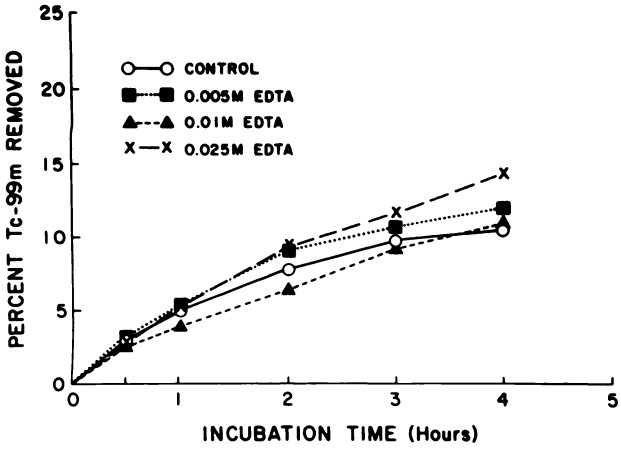

FIGURE 1
Loss of ^{99m}Tc from radiolabeled neutrophils over time. The data is reported as the mean ± s.d. of seven separate studies.

while 14.4 ± 4.8% was lost over four hours from cells incubated in 0.025*M* EDTA. Differences in the curve slopes were tested by analysis of variance (*13*), and no significant differences were found ($p > 0.05$).

Site of ^{99m}Tc Localization

In order to determine the location of ^{99m}Tc in neutrophils, we mechanically disrupted the cells and isolated the various subfractions. We discovered that ^{99m}Tc localizes in a number of different sites within neutrophils (see Fig. 2). For example, 19.8 ± 11.5% (mean ± s.d.) of the initial activity was associated with the fraction containing both nuclei and plasma membranes, 25.6 ± 3.9% was associated with mitochondria, and 26.6 ± 9.8% was associated with the microsomal fraction. In addition, a further 29.2 ± 6.9% was found in the cytosol.

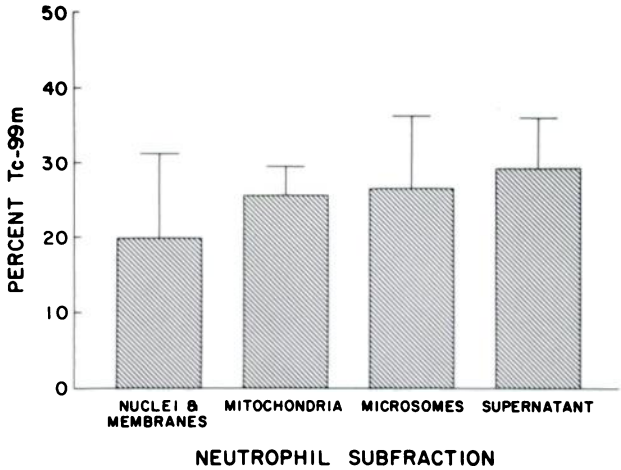

FIGURE 2
Percentage of initial ^{99m}Tc activity associated with neutrophil subfractions. The data is reported as the mean ± s.d. of 11 separate studies.

Thus, almost 70% of the activity located in cells is associated with subcellular organelles. In order to determine if ^{99m}Tc binds to protein in those organelles, we treated them with one ml of 0.1% Triton X-100 to dissolve the membranes, and then precipitated the liberated protein with TCA. In each case we discovered that from 80 to 90% of the activity was not precipitable by TCA and presumably only loosely associated with the various substructures.

In order to further quantitate the amount of radiolabel bound to neutrophil membranes, we added pepsin to a final concentration of 0.005% to 10^7 labeled neutrophils contained in one ml of Ca^{++}/Mg^{++}-free balanced salt solution (BSS) and dialyzed the preparation at 37°C against one liter of PBS containing 0.025*M* EDTA. We assumed that over time the pepsin would digest surface proteins and liberate any bound radiolabel. Nonspecifically adsorbed activity was determined by dialyzing labeled neutrophils without pepsin against 1 l of PBS containing 0.025*M* EDTA. We removed and counted 1 ml of the dialysate hourly from time 0 to 4 hr of incubation. The data were again analyzed by testing the variance of the slopes of the curves by the Student's t-test using a one-tailed criterion. In this case, untreated cells lost significantly less radiolabel over the 4-hr period than did the pepsin-treated cells ($p > 0.004$). Specifically, untreated cells lost 9.7 ± 3.3% of the initial activity over the duration of the experiment, while the pepsin-treated cells lost increasing amounts of the label over the time period, rising to 18.4 ± 3.7% at 4 hr. Thus, the pepsin-treated cells lost approximately an additional 9–10% of their activity, which presumably had been bound to surface proteins.

We also periodically tested the ability of neutrophils to exclude trypan blue during the experiment to verify that the enzyme was cleaving radiolabel from external sites and not destroying the integrity of the plasma

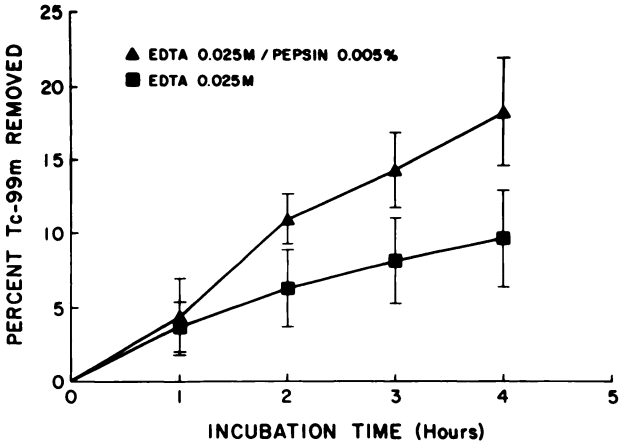

FIGURE 3
Loss of ^{99m}Tc over time from neutrophil plasma membranes by pepsin digestion. The data is reported as the mean ± s.d. of six separate studies.

membrane. With the exception of one study out of four, there was no significant difference in viability over the 4-hr period between pepsin-treated and untreated cells, as determined by the chi-squared test (Table 1).

DISCUSSION

Neutrophil radiolabeling with ^{99m}Tc has been investigated by a number of researchers as an attractive alternative method to ^{111}In labeling. To serve as a useful label for neutrophils, a radionuclide and its labeling procedure must not adversely affect cellular viability or normal function, the label should consistently bind to the cells with high efficiency, and its spontaneous release should be slow compared to the duration of the experiment. When neutrophils are radiolabeled with ^{99m}Tc, the labeling efficiency varies greatly, and we undertook the present study to better understand the reasons for this outcome.

In agreement with other investigators (*1,rc4,5*), we found that radiolabeling neutrophils with ^{99m}Tc does not affect their viability, as assessed by trypan blue exclusion, or their normal phagocytosing function. Also in agreement with other workers, we experienced considerable variability in labeling efficiency, and this variability may prove to be a major drawback to ^{99m}Tc as a radiolabel for neutrophils. Among the many parameters that have been studied in an attempt to maximize labeling efficiency, the level of stannous ion in the labeling medium is believed to be critical, and several studies have been conducted to determine the appropriate concentration of this ion. For example, in a titration study Kelbaek and Fogh (*14*) pretinned leukocytes with increasing concentrations of stannous pyrophosphate up to 200 μg/ml and obtained a maximum labeling yield of 23.7% at 30 μg/ml (18.8 μg Sn^{++}/ml), although their results were highly variable. When they studied the optimum stannous pyrophosphate concentration of 30 μg/ml, they obtained a yield of 33.5 ± 3.3%. Doly et al. (*15*) labeled ~4 × 10^7 human lymphocytes with ^{99m}Tc reduced by variable concentrations of stannous pyrophosphate and obtained maximum efficiency at 140 μg tin in a volume of 1–2 ml.

TABLE 1
Percent Viability of Radiolabeled Neutrophils at Various Times Following Treatment with 0.005% Pepsin*

	Percent viability† Incubation period (hr)		
	1	2	4
Pepsinized cells	87.4 ± 7.4	78.2 ± 9.8	80.2 ± 3.7
Control cells‡	89.2 ± 8.2	87.6 ± 2.7	83.2 ± 5.6

* Cells were dialyzed against 1,000 ml of PBS containing 0.025*M* EDTA.

† Each result represents the average of four determinations and is expressed as a mean value ± s.d.

‡ Control cells were radiolabeled with ^{99m}Tc and maintained in Ca^{++}/Mg^{++}-free balanced salt solution without pepsin treatment.

At the high extreme of stannous ion concentration, Farid's group (*1*) obtained a labeling efficiency of 81.0 ± 6.0% for human leukocytes over four experiments using 6.0 mg sodium pyrophosphate and 1.7 mg stannous chloride, although they did not report the number of leukocytes they labeled.

We chose to radiolabel rat neutrophils with a kit similar to that used by Farid et al., but our labeling efficiency was much lower and more variable than was theirs. This difference may be due to the fact that we used a different population (peritoneal) of neutrophils obtained from rats rather than neutrophils from human and canine blood used by Farid. On the other hand, our results are consistent with those of Kelbaek and Fogh (*14*) and Linhart et al. (*5*), both of whom used neutrophils from human whole blood.

Based on our experimental results, we have concluded that factors other than, or perhaps in addition to, stannous ion concentration influence the labeling yield with ^{99m}Tc. Specifically, we feel that the critical factor causing inconsistent labeling efficiency is the lack of a unique binding site for ^{99m}Tc in neutrophils. The radiolabel associates to a variable extent with several cell components with no strong affinity for any one component. We found that approximately one-half of the radioactivity associated with neutrophils was not protein-bound, and ~10% of the activity could be removed over 4 hr by dialyzing against physiological buffer. Much of this relatively mobile radioactivity could contribute to the inconsistent labeling efficiency.

This conflicts somewhat with the results of Glenn et al. (*6*) who radiolabeled leukocytes with [^{99m}Tc] pertechnetate by a stannous chloride method and performed autoradiography, which suggested the cell surface as the primary localization site. Some possible explanations for the differences between their work and ours include the fact that demonstrating the exact localization site for ^{99m}Tc with autoradiographs is difficult due to the lack of beta emissions and the unfavorable interaction characteristics of photons. Also, Glenn et al. pretinned their leukocytes with a solution of stannous chloride, while we used a stannous pyrophosphate preparation. Furthermore, the influence of pyrophosphate on localization in neutrophils is not known. Our experiments indicate that radiolabeling with stannous pyrophosphate results mainly in the intracellular deposition of label with 10%, and at most 20%, of the ^{99m}Tc that associated with neutrophils attached to the plasma membrane, while the balance associated with intracellular structures and cytosol. But regardless of the amount of ^{99m}Tc actually localized to membrane proteins, the attachment there seems to be a firm one. In preliminary work we found that EDTA can effec-

tively extract free ^{99m}Tc (or ^{99m}Tc-Sn-pyrophosphate) from a dialysis bag; on the other hand, as we have shown in the present work, EDTA is unable to liberate material bound to cell surfaces. Instead, rather harsh treatment with pepsin was required to do so.

The failure of ^{99m}Tc to associate with a single entity within neutrophils is apparently manifested in its failure to be more-routinely used in the clinical environment, a development which may have to await the discovery of a vector capable of providing high-affinity binding to one or a few specific sites.

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